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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C07H 1/00, 21/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/06985</b> <b>(43) International Publication Date:</b> 30 April 1992 (30.04.92)
<b>(21) International Application Number:</b> PCT/US91/07622 <b>(22) International Filing Date:</b> 21 October 1991 (21.10.91)  <b>(30) Priority data:</b> 598,665                      23 October 1990 (23.10.90)      US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US    598,665 (CIP) Filed on                                      23 October 1990 (23.10.90)  <b>(71) Applicant (for all designated States except US):</b> CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> SMITH, Steven, S. [US/US]; 6266 Crestwood Way, Los Angeles, CA 90042 (US). KAPLAN, Bruce, E. [US/US]; 825 North Indian Hill Place, Claremont, CA 91711 (US).  <b>(74) Agent:</b> IRONS, Edward, S.; 919 18th Street, N.W., Suite 800, Washington, DC 20006 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), NL (European patent), SE (European pat- ent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MECHANISM BASED INHIBITORS OF DNA METHYLTRANSFERASE  <b>(57) Abstract</b>  Method for producing double stranded oligonucleotide compounds by providing a self-priming DNA fragment and ex- tending said double stranded DNA with a DNA polymerase and with a combination of dNTP's and 5-fdCTP, and compounds directed to same.		

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MECHANISM BASED INHIBITORS  
OF DNA METHYLTRANSFERASE

This application is a continuation-in-part of United States application Serial No. 598,665 filed 23 October, 1990, which is a continuation of United States application Serial No. 317,670 filed 1 March 1989, which in turn is a continuation-in-part of Serial No. 314,935 filed 24 February 1989.

FIELD OF INVENTION

This invention relates to synthetic oligomers of unique three-dimensional structure that combine the principles of polymer, peptide and synthetic DNA chemistry to provide rationally designed drugs, drug delivery systems, research tools and other products.

The invention also relates to unique oligonucleotide molecules in which 5-fluorodeoxycytidine inhibits DNA methyltransferase.

BACKGROUND OF THE INVENTION

Various heteropolymers are known (see, e.g., Seela, F. and Kaiser, K., Oligodeoxyribonucleotides containing 1,3-propanediol as a nucleoside substitute, Nucleic Acids Res. 15:3113-3129 (1987) and Connolly, B.A., The Synthesis of Oligonucleotides containing a primary amino group at the 5'-terminus, Nucleic Acids Res. 15:3131-3139 (1987)) and one example of a heterodimer exists. See Lemaitre, M., Bayard, B., and Leblue, B., Specific Antiviral Activity of a Poly(L-lysine)-Conjugated Oligodeoxyribonucleotide Sequence Complementary to

Vesicular Stomatitis N Protein mRNA Initiation Site,  
Proc. Nat. Acad. Sci. U.S.A. 84:648-652 (1987).

However, there is no known disclosure of molecules comprising linked heterologous blocks conforming to a predetermined or predictable three-dimensional structure.

#### Definitions

The following definitions apply to terms used in this specification and in the claims:

Block--An oligomer component of at least about 5 monomers, e.g., carbon atoms, amino acid residues or nucleotides which has an intrinsic tendency to self-associate inter se or with another block.

Unit--An oligomer component consisting of at least three blocks.

Oligomer--A molecule having from about 12 to about 150 monomers, e.g., carbon atoms, amino acid residues or nucleotides and comprising at least one unit.

Self-Association--The capability shared by two or more blocks to form a mutual linkage, e.g., the capability of homologous nucleic acid sequences to hybridize and of certain peptides to interact.

Homologous Block--One of a series of blocks where members of the series exhibit common properties, for example, one of a series of nucleic acid, peptide or organic polymer blocks. Organic polymer blocks may generally comprise straight or branched chain polyolefins such as polyethylene, polypropylene or polystyrene and other kinds of polymers which are not cross-linked.

Heterologous Block--One of a series of blocks whose members do not exhibit common properties.

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Heterologous Block Oligomer (HBO)--An oligomer comprising at least one unit of the schematic formula  $A_1-B-A_2$  in which the block B is heterologous with respect to at least one of the blocks  $A_1$  and  $A_2$  and is constrained into a generally looped configuration by the self-association energy of the blocks  $A_1$  and  $A_2$  or in which the blocks  $A_1$  and  $A_2$  are heterologous and are constrained into a spatially juxtaposed position by the internal self-association of the block B.

#### SUMMARY OF THE INVENTION

HBO's provide a broad spectrum of novel molecules. The new molecules may be predesigned to achieve objectives which have been realized, with some difficulty, if at all.

HBO detergents can be formed with self-associated DNA blocks joined by simple linker blocks. The properties of these detergent molecules can be exploited in several ways. Micelles formed primarily of the self-associated DNA blocks may permit the passage of any short, double-stranded DNA through the bloodstream. Antisense molecules and short duplex DNA's more intrinsically resistant to DNase could be delivered in this fashion. "Suicide" substrates of duplex DNAs can be constructed to target tissues and neoplastic or virus infected cells. The inclusion of a hydrophobic linker block in these HBOs facilitates diffusion or transport across cell membranes at the requisite site.

A variety of applications in protein purification, for example, hydrophobic interaction chromatography are apparent. More specifically the biospecificity of DNA sequences may combine with the

capacity of the linker block to interact with such a chromatographic column.

Both 5-fluorodeoxycytidine (FdCyd) and 5-azacytidine (azaC) have been shown to inhibit methylation of DNA with resultant effects on gene expression and cell differentiation. See, e.g., Jones and Taylor, Cell 20:85-93 (1980) and Osterman et al., Biochemistry 27:5204-5210 (1988). Instability is a major difficulty with azaC which hydrolyzes readily. Incorporation into DNA appears to increase stability to some extent. However, isolation of intact azaC containing DNA is difficult. See, Santi et al., Proc. Natl. Acad. Sci. USA 91:6993-6997 (1984).

In contrast, FdCyd and DNA containing FdCyd are stable to chemical hydrolysis. FdCyd, however, is susceptible to enzymatic deamination to toxic 5-fluorodeoxyuridine metabolites. See, Newman et al., Proc. Natl. Acad. Sci. USA 79:6419-6423 (1982).

Attempts to prepare synthetic oligonucleotides which contain FdCyd in appropriate positions corresponding to the methylation sites of DNA-cytosine methyltransferase have failed. See, Osterman, et al., supra. Hence resort was had to an enzymatic synthesis of poly (FdC-dG). Ibid. Hardy et al, Biochemical and Biophysical Research Communications, 145, 146-152 (1987) describe M13 DNAs in which carbon-5 of each deoxycytidine residue in one chain is replaced by a bulky group. These DNAs are shown to be good substrates for human DNA (cytosine-5) methyltransferase. These substituted DNA's were prepared by using 5 MedCTP, 5 BrdCTP, 5 IdCTP and 5 FdCTP in place of dCTP during primer extension as described in Messing J., Methods in Enzymology 101:20-78 (1983).

This invention provides self-priming HBOs in which (1) the blocks  $A_1$  and  $A_2$  are homologous DNA sequences (2) the block designated  $A_2$  contains fewer, e.g., at least 10 fewer bases, and preferably from about 10 to about 50 fewer bases, than the block designated  $A_1$ , and in which the block  $A_2$  is a primer for the block A and may be extended, for example, by a DNA polymerase with a combination of dNTPs and FdCTP. Such an HBO is depicted, for example, by the molecules designated 48L-1, 48L-2 and 48L-3 in Table 1, *infra*. This invention further provides synthetic oligonucleotide molecules produced by the extension of such a self-priming HBO with a combination of dNTPs and FdCTP. Such molecules are mechanism based inhibitors of human DNA methyltransferase.

#### DESCRIPTION OF THE FIGURES

Figure 1 is a two-dimensional generalized schematic depiction of an HBO in which the  $A_1$  and  $A_2$  blocks are self-associated.

For example, in such an HBO, the  $A_1$  and  $A_2$  blocks may be self-associating oligonucleotides, peptides or the like and the linker block B provides a preselected property, e.g., hydrophobicity. Alternatively, the linker block B may be nucleic acid sequences when the  $A_1$  and  $A_2$  blocks are self-associating peptides.

Figure 2 depicts an HBO in which the linker block B is internally self-associating, e.g., a homologous DNA sequence, flanked by  $A_1$  and  $A_2$  blocks such as peptides or organic polymers which do not self-associate and which are constrained in juxtaposition by the self-association energy of the



linker block. Suitable DNA sequences for the B blocks of such HBOs include a stretch of hybridized nucleotides, generally a sequence of about 15 to 50 bases to provide the self-association energy appropriate to maintain the desired juxtaposition of the A<sub>1</sub> and A<sub>2</sub> blocks. Suitable peptides for use as A blocks contain from about 5 to about 30 residues. Suitable A block polymers include RNA, DNA, peptides or mixed RNA-DNA polymers having 12 to 150 nucleotides.

Figure 3 is a copy of an autoradiograph illustrating the utility of an HBO as a human methyl transferase substrate.

Figure 4 illustrates a three nucleotide rule DNA methylation with DNA methyl transferase.

Figure 5 depicts the Methyl-Directed DNA Methyltransferase Reaction.

Figure 5A: Space-filling models of the asymmetrically methylated B-DNA molecule and the symmetrically methylated B-DNA molecule produced by the enzyme, depict the reaction. Methyl groups on 5-methylcytosine residues at the CG dinucleotide pair are shown.

Figure 5B: Restriction analysis of the enzymatically labelled product. Labelling pattern predicted for the product of the methyl-directed reaction is shown on the left. The position of the 5-methylcytosine residue introduced synthetically is indicated with an (m); the predicted position of the 5-methylcytosine generated enzymatically is indicated with an (\*). Cleavage sites for the indicated restriction enzymes are depicted as gaps. the autoradiograph on the right shows the sizes of the labelled restriction fragments observed after enzymatic labelling with [<sup>3</sup>H-methyl]AdoMet.

Figure 6 depicts consequences of the Enzyme Mechanism.

Figure 6A: Mechanism of Human DNA(cytosine-5) methyl-transferases: Nucleophilic attack at C-6 of cytosine by a group at the active site of the DNA. Methyltransferase activates C-5 of cytosine as a methyl acceptor by saturating the 5-6 double bond. AdoMet donates a methyl group to C-5 of cytosine to form AdoHcy and the bracketed dihydrocytosine intermediate shown. Dissociation of the enzyme from the complex results in the regeneration of the 5-6 double bond to produce 5-methylcytosine. In the alternate pathway shown below the brackets, the protonated form of the dihydrocytosine intermediate undergoes hydrolytic deamination to generation dihydrothymine (bottom center). Dissociation of the enzyme from this intermediate results in the regeneration of the 5-6 double bond to form thymine.

Figure 6B: Stable Enzyme-DNA Complex with 5-FC: C-5 of 5-FC, can also be activated as a methyl-acceptor through nucleophilic attack by the DNA methyltransferase at C-6. However, transfer of the methyl-group from AdoMet results in a stable covalent complex between DNA and the enzyme, because neither the C-F nor the C-CH<sub>3</sub> bond at C-5 can be broken to regenerate the 5-6 double bond in the cytosine ring.

Figure 7 depicts mechanism-based labelling of the human DNA(cytosine-5)methyltransferase.

Figure 7A: As starting material, a 47mer was synthesized from  $\beta$ -cyanoethylphosphoramidite precursors using an ABI DNA synthesizer. The 47mer was constructed so as to link a long block of DNA to a shorter complementary block of DNA through a tether

consisting of five thymine residues. A 5-methylcytosine residue was introduced during synthesis at position 17 (indicated with m). Molecules of this type form unimolecular foldbacks and are self-priming substrates for DNA polymerase I. In order to generate an oligodeoxynucleotide in which position 48 was occupied by 5FC, 5-FdCTP synthesized by the method of Ruth, et al. Mol. Pharm. 20:415 (1981) was used in place of dCTP during extension with the Klenow fragment of DNA polymerase I, placing 5FC at residues (indicated with F) between positions 48 to 65 normally occupied by C. The FdC residue at position 48 is indicated with an asterisk. Extension to full length was demonstrated by gel electrophoresis on 20% polyacrylamide sequencing gels. The product 65mer was purified by HPLC on a FACS anion exchange column (Waters Associates, Medford, Mass). The presence of 5-FdC, 5mdC, and other deoxynucleosides in appropriate stoichiometry was demonstrated by HPLC analysis of the nucleoside produced after digestion of the 65mer with nuclease P1 and bacterial alkaline phosphatase using the method of Palmgren, et al. Biochim. Biophys. Acta 1049:293 (1990).

Figure 7B: Primer extension products were end-labelled using T4-polynucleotide kinase and incubated with a crude DNA methyltransferase preparation under the conditions given below. The product was concentrated by precipitation with trichloroacetic acid and resuspended in buffer containing  $\beta$ -mercaptoethanol and sodium dodecylsulphate. After heating to 95°C for 5 minutes the sample was separated by electrophoresis through sodium dodecylsulphate containing polyacrylamide

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gels.  $^{32}\text{P}$  was visualized by autoradiography. Lane 1: 5FdC-65mer + complete reaction mixture : 50 mM 4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid (pH 7.0) (HEPES), 50 mM NaCl, 2 mM dithiothreitol, 75  $\mu\text{M}$  spermine, 10% (v/v) glycerol, and 6.0  $\mu\text{M}$  [ $^3\text{H}$ -methyl]AdoMet. Lane 2: 5FdC-containing 65mer + reaction mixture lacking AdoMet. Lane 3: dC-containing 65mer + complete reaction mixture.

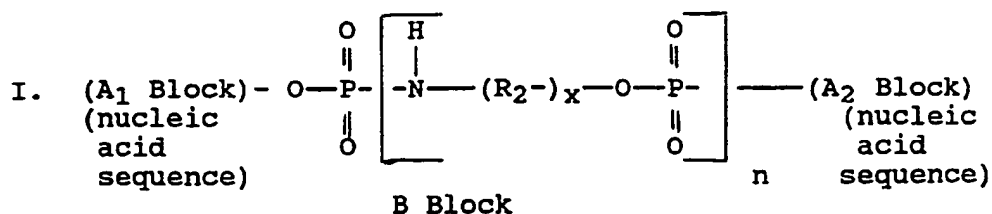
Figure 7C: Unlabelled primer extension products were incubated with the methyltransferase preparation and separated by electrophoresis through sodium dodecyl sulphate-polyacrylamide gels as in A except that the tritiated methyl-groups applied to the foldback were visualized by fluorography after impregnating the gels with En $^3$ Hance. See, Smith, et al., *J. Mol. Biol.* 217:39 (1991). Lane 1: complete reaction mixture + 5FdC-containing 65mer. Lane 2: Complete reaction mixture + dC-containing 65mer.

Figure 8 depicts chromatographic Analysis of Enzymatically Labelled DNA.

A duplex oligodeoxynucleotide 30mer (inset) was enzymatically methylated in the complete reaction mixture containing [ $^3\text{H}$ -methyl]AdoMet labelled in the methyl-moiety. After digestion of the labelled oligodeoxynucleotide with nuclease P1 and bacterial alkaline phosphatase, the liberated nucleosides were separated by HPLC on a  $\mu$  bondapack C18 column. (Palmgren et al, *Biochem. Biophys. Acta* 1049:293 (1990)) Fractions were collected and tritium was quantified by scintillation counting. 8(A) Tritium profile. 8(B) Absorbance profile.

Formula I schematically represents one form of an HBO of the kind depicted by Figure 1:

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in which R is an alkyl or aryl group of from about 1 to about 10 carbon atoms and x may be from about 3 to about 12. When x is greater than about 10, these HBO's are surfactive.

The number, n, of B block moieties depends upon the properties desired in the HBO. For many purposes n is from about 5 to about 20.

Appropriate selection of the A<sub>1</sub>, B and A<sub>2</sub> blocks yields bioengineered catalysts in which catalysis is carried out by an appropriately constrained peptide, protein or RNA block. In addition, hydrophobic B blocks, e.g., amino-alkyl phosphonates, amino-aryl phosphonates, yield HBOs which are surfactants, particularly when the self-associating blocks are DNA.

#### EXEMPLIFICATION OF THE INVENTION

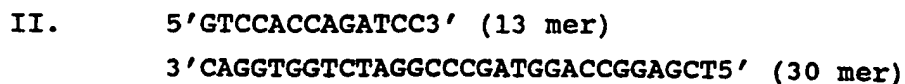
##### EXAMPLE I

This example demonstrates the scope and significance of the invention. To do so, it identifies a specific question which has arisen in scientific research, describes the design of an HBO for use in answering the question, exemplifies the synthesis of the postulated HBO and shows that the synthesized HBO functions as intended.

The question addressed concerns the substrate specificity of the human DNA methyltransferase, i.e., whether the enzyme is capable of methylating across a gap in duplex DNA.

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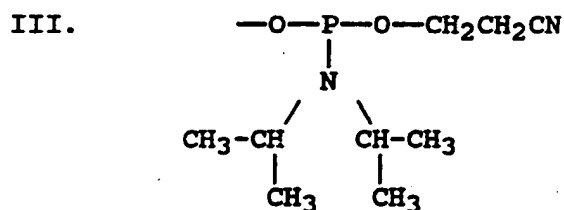
A 30 mer and a 13 mer were selected to provide a gapped duplex DNA as depicted by Formula II:



Prior experience indicated that the Formula II molecule might be unstable due to the short region of homology. Stability was achieved by linking the 13 mer and the 30 mer by a block effective to promote the annealing under the conditions contemplated for the reaction, i.e., 37°C, 50 mM HEPES, pH7.1, 70  $\mu$ M Spermine, 1.5  $\mu$ M 5-adenosyl-L-(methyl-<sup>3</sup>H) methionine [<sup>3</sup>H AdoMet].

The design of the linker block raised two questions: (1) what moiety should be used to construct it, and (2) how long should it be? Hydrophobic moieties were rejected to preclude any test of the capability of the enzyme to interact with a detergent.

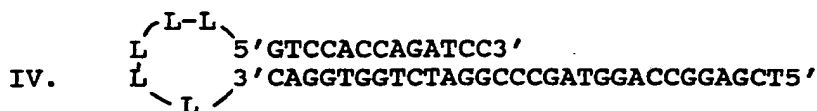
Cyanoethyl disopropyl phosphoramidite



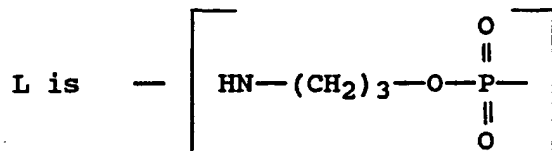
was chosen as the linker moiety because it is appropriately protected for phosphoramidite synthesis and hence could be added to the termini DNA strands of the Formula II duplex.

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Computer modelling in which the desired molecule was constructed in BIOGRAPH was used to select the length of the linker group. Monomeric methane moieties were condensed to the 5' phosphate oxygen of the 13 mer until a methylene chain  $(-CH_2-)_n$  long enough to reach the 3' hydroxyl of the 30 mer without producing a conformational change in the Formula I duplex DNA was constructed. The minimum length was determined to be 24  $(-CH_2-)$  moieties. The selected linker monomer is somewhat longer than such moieties. Accordingly, a linker block consisting of five linker units "L" was selected. The HBO to be synthesized is illustrated by Formula IV:



in which



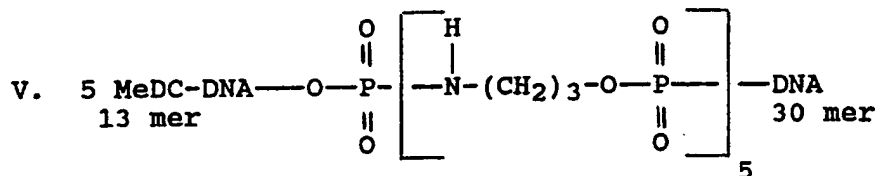
#### Oligodeoxynucleotide Preparation and Characterization

Single strand oligodeoxynucleotides were synthesized using the phosphoramidite method (Sinha, N.D., et al., Nucleic Acids Res. 12:4539-4557 (1984)). The single stranded products were purified by polyacrylamide gel electrophoresis and high performance liquid chromatography as described by Tan, et al., Cold Spring Harbor Symp. on Quantitative Biology XLVII 383-391 (1982). The sequence of each of the oligodeoxynucleotides was verified using the method of Maxam and Gilbert.

### Duplex Oligode xynucleotides

The HBO was synthesized in a manner similar to the standard production of oligodeoxynucleotides. The amino group is protected by a monomethoxyl trityl MMT or dimethoryl trityl DMT group and the phosphate group is simultaneously activated. After the MMT or DMT group was removed, the amino group was neutralized after each addition so that the next monomer could be added.

Formula V schematically depicts the HBO produced by this example:



The HBO product of this example is an excellent substrate for human DNA methyltransferase as evidenced by the following test which is dependent on the spatial conformation of the molecule.

DNA(cytosine-5)methyltransferase was purified from human placentas as described in Smith, supra. When stored under the conditions described there, the enzyme loses less than 50% of its activity per year at -70°C. Two sets of assay conditions were employed. The unit of enzyme activity is defined in terms of the assay conditions used during enzyme purification with heat-denatured Micrococcus lysodeikticus DNA substrate (Smith supra). A unit of enzyme activity is the amount required to catalyze the incorporation of 1 pmole of methyl groups into TCA insoluble DNA in one hour at 37°C under those conditions.



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For the determination of initial velocities with oligodeoxynucleotide substrates, the enzyme was dialyzed for 3 hours in a Hoefer microdialyzer (Health Products Inc., Rockford, Ill.) against 38 mM glycine, 17% v/v glycerol, 5 mM Tris, 10 mM  $\beta$ -mercaptoethanol, pH 7.8 at 4°C. The final reaction volume of 100  $\mu$ l contained: 0.4  $\mu$ g total DNA, 50 mM HEPES pH 7.0, 50 mM NaCl, 2 mM DTT, 75  $\mu$ M Spermine, 10% v/v glycerol and 6.0 mM [ $^3$ H]AdoMet (Amersham, 15 Ci/mmol). Reaction mixtures were pre-incubated at 37°C for 15 minutes before the addition of 44 U of DNA methyltransferase to initiate the reaction. The reaction rate was linear under these conditions for 30 minutes. After 20 minutes of incubation, the reactions were stopped by the addition of 5 ml of cold TCA (5% w/v TCA containing 5 mM potassium pyrophosphate). Tritium incorporated into TCA insoluble DNA was determined as previously described (Smith, S.S., supra).

After enzymatic methylation of the substrate molecules with [ $^3$ H]5-adenosyl methionine as methyl donor, the labelled duplexes were cleaved with restriction endonucleases MboI and MspI. The products were electrophoretically separated and  $^3$ H labelled DNA fragments were detected by fluorescence enhanced autoradiography as previously described in Smith, et al. The HBO molecule is refractory to digestion by MspI, consistent with the fact that the looped molecule cannot generate a complete duplex MspI site. On the other hand the same molecule is cleaved by MboI to about 70% completion. The cleavage product is only slightly shorter than the uncut molecule suggesting that MboI cleavage occurs on the cut site on the unmethylated portion of the

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molecule to produce a molecule that is six nucleotides shorter (on the 3' end) than the parent molecule. This partial cleavage pattern could be produced by the presence of the linkers in the loop, or it could mean that the fold-back structure does not form in a way that provides the enzyme with a completely recognizable cleavage site.

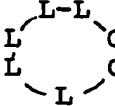
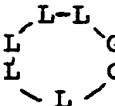
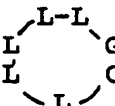
In any event, the DNA methyltransferase recognizes the structure and actively methylates it. The data given in Table I shows that the presence of a methyl group at the end of the short arm of the loop stimulates the reaction more than 100 fold (48L-1), while the presence of the methyl group at position 17 (bases numbered from the 5' end of the molecule) (48L-2) does not stimulate the reaction rate.

These findings are consistent with a three nucleotide rule for DNA methylation which may be elucidated by reference to Figure 7. Referring to the figure, if enzymatic methylation occurs at cytosine (3), only the nucleotides shown in the L-shaped box are required. The enzyme must interact with cytosine (1) and its hydrogen bonded guanine (2) as it methylates cytosine (3). Guanine (4) is not required and can in fact be missing, alkylated, or replaced with any base. Even when the structure of a DNA substrate is not well understood, the application of this "L-Test for Methylation" or "3 nucleotide rule" is useful in predicting the methylation pattern applied in vitro.

It clearly demonstrates that the DNA molecule that we have constructed is a well behaved enzyme substrate.

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TABLE I

<u>Molecule Code</u>	<u>Structure</u>	<u>DNA Methyl- transferase Substrate Activity (FMOLE/MIN)</u>
48L-1	 GTCCACCAGATCC3' M CAGGTGGTCTAGGCCCGATGGACCGGAGCT5'	157
48L-2	 GTCCACCAGATCC3' CAGGTGGTCTAGGCCCGATGGACCGGAGCT5' M	11
48L-3	 GTCCACCAGATCC3' CAGGTGGTCTAGGCCCGATGGACCGGAGCT5'	12

Enzymatic methylation of the HBO 48L-2 is demonstrated by the strong tritium signal in lanes 2 and 3 of the Figure 3 autoradiograph. To produce the autoradiograph HBO 48L-2 was exposed to human DNA methyltransferase in the presence of S adenosyl methionine as the methyl group donor. The reaction product was separated by polyacrylamide gel electrophoresis and the presence of enzymatically tritiated DNA was demonstrated by fluorescence enhanced autoradiography. Referring to Figure 3, lanes 1 and 7 include markers corresponding to 18, 24 and 30 bases. MboI and MspI identify restriction enzymes.

HBO's of all types may be synthesized by techniques known to the skilled person. Linker blocks B may be added to pre-self associated A<sub>1</sub> and

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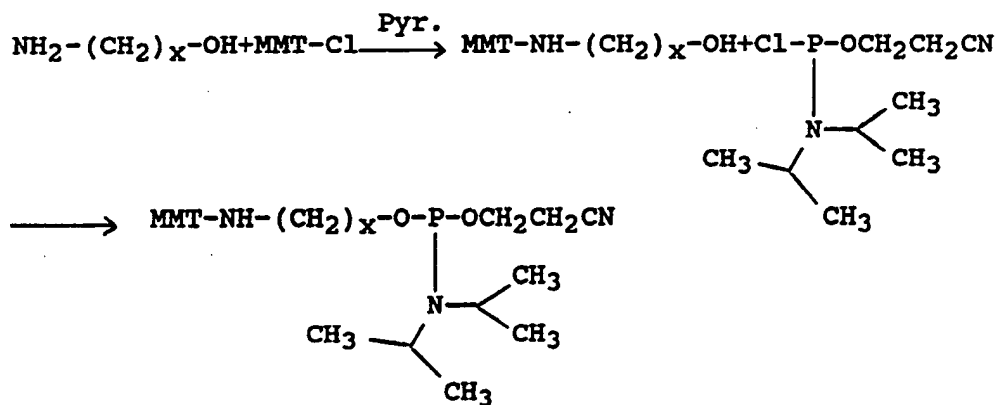
A<sub>2</sub> blocks as typified by the example. Alternatively, all of the blocks may be separately synthesized and the desired HBO constructed from these prefabricated blocks. Such a procedure is preferred for the production of HBO's which involve the linkage of peptide and DNA blocks.

### EXAMPLE II

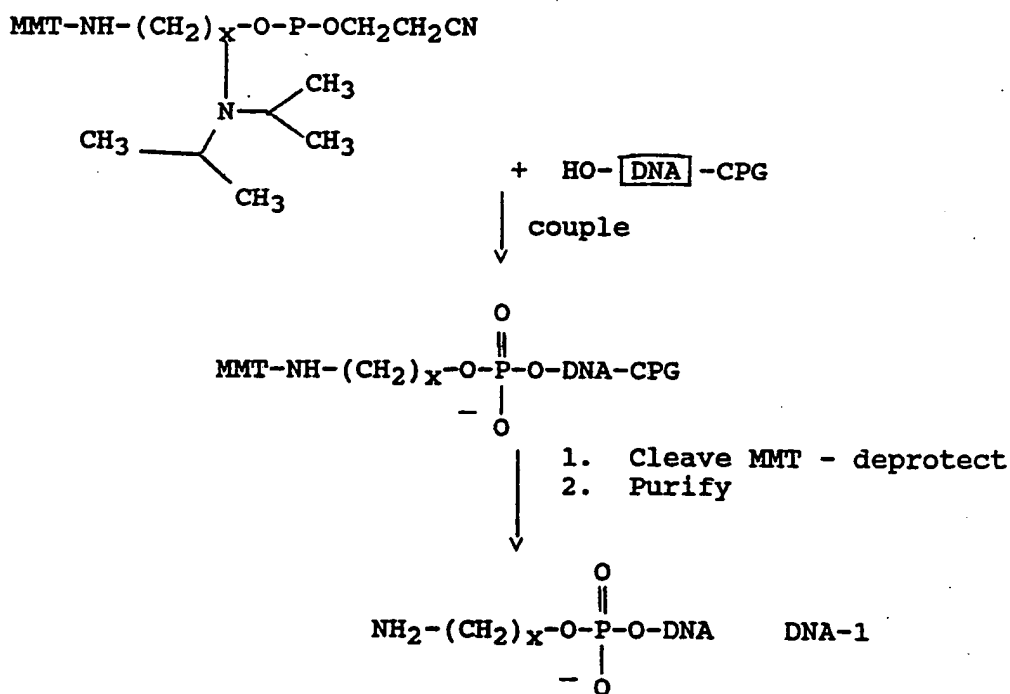
Example II illustrates one method for producing an HBO of schematic formula (DNA-2)-peptide-(DNA-1).

#### Synthesis of DNA-1

An amino alcohol is reacted with one equivalent of monomethoxytrityl chloride (MMT-Cl) in pyridine. The MMT-amino alkanol product is purified by chromatography and then reacted with an appropriate phosphitylating agent forming a cyanoethyl-diisopropylamino phosphite or a hydrogen phosphonate reagent or any other phosphite reagent known to the skilled worker. The product of this reaction is an activated phosphite reagent useful in any standard DNA synthesis machine. These reactions are illustrated by the following equation:

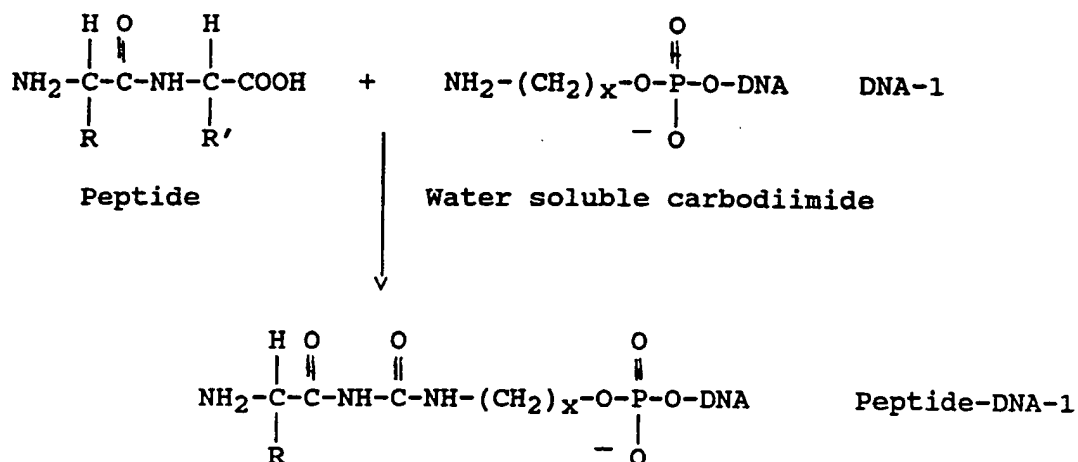


This activated phosphate reagent is coupled to the 5'OH of a growing DNA molecule synthesized in known manner on a solid support such as controlled pore glass (CPG). The MMT group is then removed with dichloroacetic acid or trichloroacetic acid and the amino group is neutralized to permit coupling to the next incoming phosphite reagent. Neutralization is accomplished by treating the growing DNA molecule with a dilute basic solution such as 1% triethyl amine in acetonitrile for a few seconds to convert the protonated amino group into a free amino group. These reactions are illustrated by the following equations:



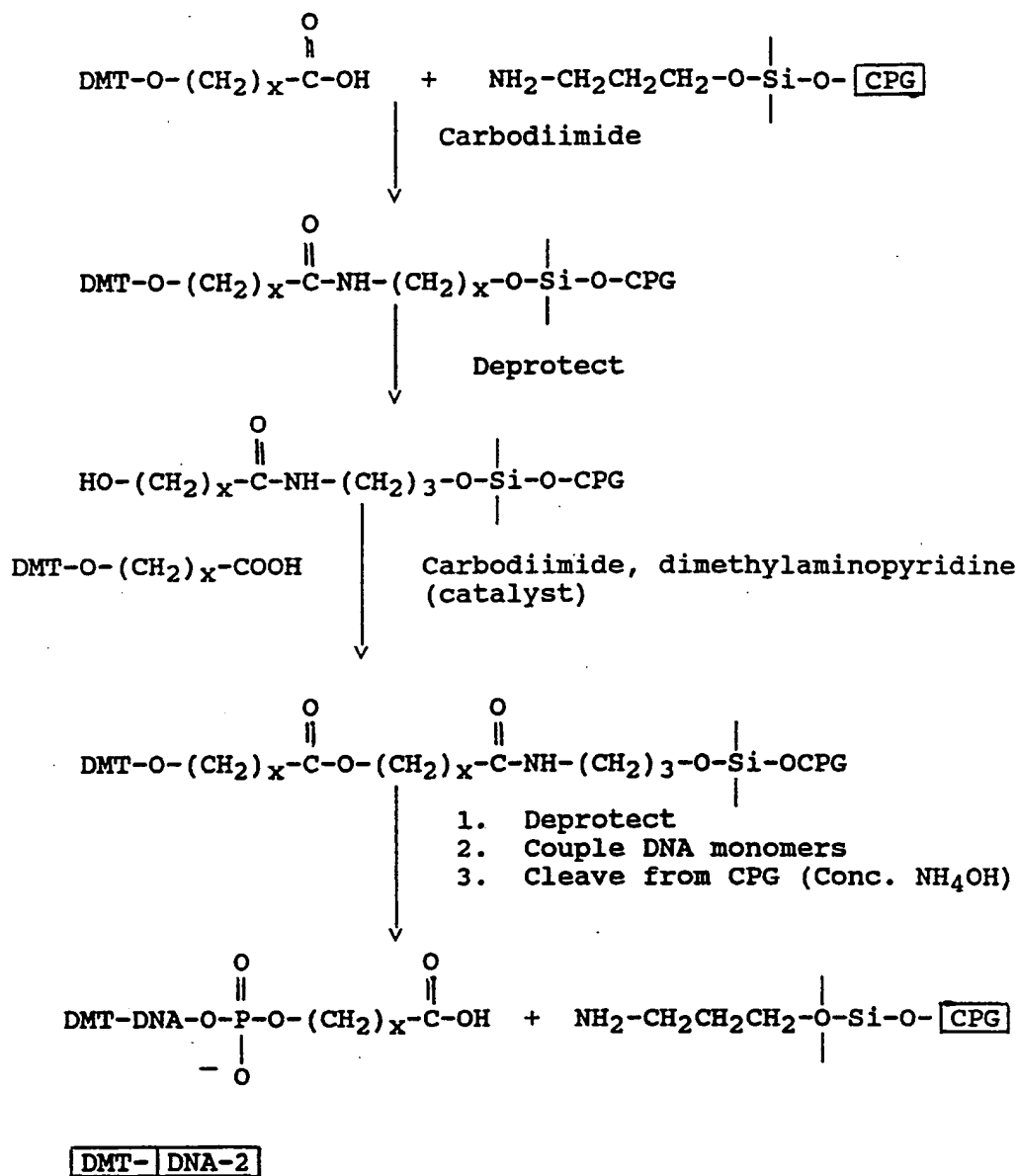
Synthesis of Peptid DNA-1

A peptide having a free amino and a free carboxyl group is coupled to DNA-1 in known manner, e.g., by use of a water soluble carbodiimide as illustrated by the following equation:

Synthesis of DNA-2

A DNA fragment with a free carboxylic acid on the 3' end is synthesized on a solid support, for example, by connecting a DMT protected hydroxy carboxylic acid such as the DMT protected 6-hydroxyhexanoic acid to an amino-propyl CPG using a carbodiimide. After the coupling, the DMT group is removed in known manner using dichloroacetic acid in dichloromethane. A second reaction with a DMT protected hydroxy carboxylic acid is completed. The DMT group is again removed and again coupled with the DMT-protected 6-hydroxyhexanoic acid using a carbodiimide and dimethylaminopyridine to provide controlled pore glass as a support for the synthesis of DNA-2 in known manner. These reactions are illustrated by the following equations:

-20-



#### Synthesis of (DNA-2)-Peptide-(DNA-1)

After the synthesis is completed, the DNA-2 is deprotected in the standard way yielding a 5' DMT-DNA-2. The 3' carboxyl of this molecule is

-21-

coupled to the amino group of the peptide-DNA-1 and the DMT group is removed to yield as the final product DNA-2-peptide-DNA-1.

5' DMT-DNA-2 + Peptide-DNA-1



DMT-DNA-2-peptide-DNA-1



Deprotect DNA

DNA-2-peptide-DNA-1

### EXAMPLE III

Example III illustrates the preparation of an oligonucleotide containing 5FC residues. This oligonucleotide is shown to be a mechanism based inhibitor of DNA methyl transferase.

As shown by Figure 5A, methylated pyrimidines are generated from the dihydropyrimidine intermediates by elimination of the hydrogen atom at C5 and the enzyme moiety at C6 to regenerate the 5-6 double bond. This should not be possible when the pyrimidine being attacked is 5FC, because the enzyme cannot break the F-C bond. For this reason, nucleophilic attack and methyl-group transfer at a 5FC residue will produce a stable complex between DNA and the enzyme (Figure 5B).

To detect the putative intermediate, the enzymatic methylation of the asymmetrically methylated foldback depicted in Figure 5B was studied. Restriction analysis of the enzymatically tritiated product using previously established methods (see, Smith, et al., J. Mol. Biol. 217:39 (1991)) showed that the 5-methylcytosine residue at



position 17 directed the enzyme to methylate the cytosine residue located at the symmetrically placed site in the CG dinucleotide pair (position 48). A rate enhancement of about 100 fold over the rate observed with the unmethylated foldback was coupled with a very strong specificity for the cytosine at position 48 (Figure 5B). This specificity was then exploited by producing an oligodeoxynucleotide containing 5FC at position 48. To do this, DNA polymerase I was used to extend a self-priming 47mer in the presence of 5FdCTP as shown in Figure 7A.

When the  $^{32}\text{P}$  end-labelled oligodeoxynucleotide was incubated with partially purified DNA methyltransferase and S-adenosylmethionine (AdoMet) covalent complexes could be detected as slow moving bands in sodium dodecylsulfate (SDS) polyacrylamide gels. The complexes survived 5 minutes at 95°C in SDS and the reducing agent  $\beta$ -mercaptoethanol (Figures 7B and 7C). The major complex had an apparent molecular weight of 160kD, which is consistent with a complex formed between the 24kD oligodeoxynucleotide and the 126kD catalytic unit obtained in homogeneous form by further purification. See, Zucker, et al., J. Cell. Biochem. 29:337 (1985). The complexes with apparent molecular weights of 130kD and 90kD could correspond to the 105kD and 79kD forms of the placental enzyme seen in immunoprecipitation experiments. See, Pfeiffer, et al., J. Biol. Chem. 260:13787 (1985). Complexes were not formed in the absence of AdoMet suggesting that the transfer of a methyl group to DNA was required for their formation. The complexes were not observed with a control oligodeoxynucleotide prepared by primer extension with dCTP in place of 5FdCTP.

Tritium fluorography could also be used to visualize the complexes when methyl groups were transferred to DNA by the enzyme from [<sup>3</sup>H-methyl]AdoMet (Figure 7C). When the 5FC containing oligodeoxynucleotide was used, more than 80% of the label was observed in complexes. The small amount of label at the position of the oligodeoxynucleotide 65mer could be attributed to nonspecific breakdown of the complex. When the oligodeoxynucleotide 65mer lacking 5FC was incubated with the enzyme and [<sup>3</sup>H-methyl]AdoMet, no label was observed in complexes. The bulk of the tritium label was observed at the position of the 65mer. Based on the specific activity of the homogeneous enzyme, about 20% of the available DNA methyltransferase was converted into stable complexes in 30 minutes with the 5FC containing oligodeoxynucleotide.

Incorporation into trichloroacetic acid insoluble material was about 40-fold lower when 5FC was present in the oligodeoxynucleotide than when it was absent, indicating that the reaction with the 5FC-containing oligodeoxynucleotide was stoichiometric while that with the oligodeoxynucleotide lacking 5FC was catalytic. More specifically, 9800 cpm/pmole tritiated methyl groups, when AdoMet is spotted directly onto a glass fiber filter and counted in scintillation fluid, is observed. The specific activity of homogeneous enzyme is about 40,000 U/mg, its molecular weight is about 126kD. When 5.7 U of enzyme (143ng) was incubated with an excess of 5FC-containing DNA, 2,212 cpm <sup>3</sup>H was incorporated into trichloroacetic acid insoluble material. If it is assumed that one mole of substrate bound per mole of enzyme, this implies that (2,212cpm/9800cpm/pmole)

-24-

(126000pg/pmole)=28.4ng of enzyme is contained in the complex, for an efficiency of about 20% (28.4/143).

The data described above indicates that the enzyme operates via the mechanism described in Figure 6. Several of the consequences drawn based on the assumption of the formation of this intermediate by eukaryotic methyltransferases have been discussed. See, Smith, et al. J. Mol. Biol. 217:39 (1991) and Santi, et al. Cell 33:9 (1983). The potential for hydrolytic deamination during catalysis has not yet been explored however. It has sometimes been noted that MspI digests like that shown in Figure 5 exhibit a small residue of enzymatically methylated DNA that is uncleaved. Since the G/T mismatch that would be generated by hydrolytic deamination as described in Figure 6A would block MspI cleavage, possibility that a low level of deamination might occur during enzymatic methylation was investigated. After enzymatic labelling, the tritium-labelled oligodeoxynucleotide was digested to deoxynucleosides and the digest was analyzed by HPLC. A comparison of the tritium profile and the absorbance profile in Figure 5 shows that tritium-labelled methyl groups were detected in 5-methyldeoxycytidine but not in thymidine. Assuming that the limit of detection is about 50% above background, calculation shows that less than 1 thymidine would be produced for every 20,811 cytosine residues methylated. More specifically, the average cpm for the 5-methylcytosine peak was  $9.78 \times 10^5$ . Average background in the region of thymidine peak was 93 cpm. Assuming a detection limit of 50% above background:  $9.78 \times 10^5 / 47 = 20,811$ . This means in vitro fidelity observed with the human DNA

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methyltransferases in copying a methylation pattern would be comparable to the fidelity ( $1/2930$  to  $1/47,500$ ) observed for mammalian polymerases in copying a DNA sequence. See, Loeb, et al. Ann. Rev. Biochem. 52:429 (1982). Since there is no apparent reason to suspect from the data that thymidine residues were actually produced during the reaction, the fidelity of the methyltransferase may in fact be considerably better than that of the mammalian polymerases.

## WE CLAIM:

1. A self-priming HBO in which the blocks  $A_1$  and  $A_2$  are homologous DNA sequences and in which one of said blocks  $A_1$  and  $A_2$  contains fewer bases than and functions as a primer for the other of said blocks and may be extended by a DNA polymerase with a combination of dNTPs and FdNTPs.

2. A self-priming HBO as defined by claim 1 in which one of said blocks  $A_1$  and  $A_2$  which contains at least 10 fewer bases and functions as a primer for the other of said blocks and may be extended by a DNA polymerase with a combination of dNTPs and FdNTPs.

3. A self-priming HBO as defined by claim 1 in which one of said blocks  $A_1$  and  $A_2$  which contains from at least 10 to about 50 fewer bases and functions as a primer for the other of said blocks and may be extended by a DNA polymerase with a combination of dNTPs and FdNTPs.

4. A method for producing a synthetic, double stranded oligonucleotide molecule as defined by claim 1 which comprises providing a self-priming DNA fragment having first and second strands said second strand containing fewer bases than and being a primer for said first strand and extending said second strand with a DNA polymerase and a combination of dNTP's and 5-FdCTP.

5. A method as defined by claim 1 in which said second strand contains at least ten (10) fewer bases than said first strand.

6. A method as defined by claim 1 in which said first and second strand are connected at their respective 3' and 5' termini by a linker to form an HBO.

FIG. 1

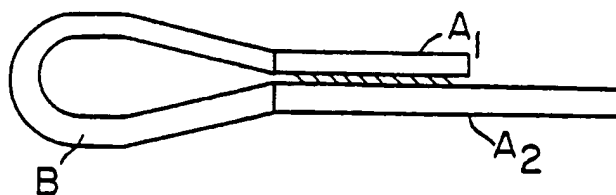


FIG. 2

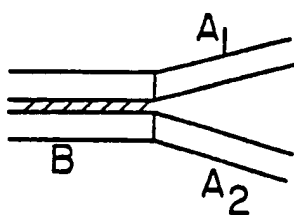
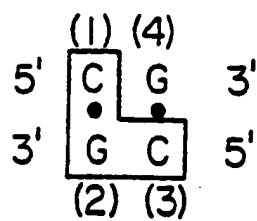


FIG. 4



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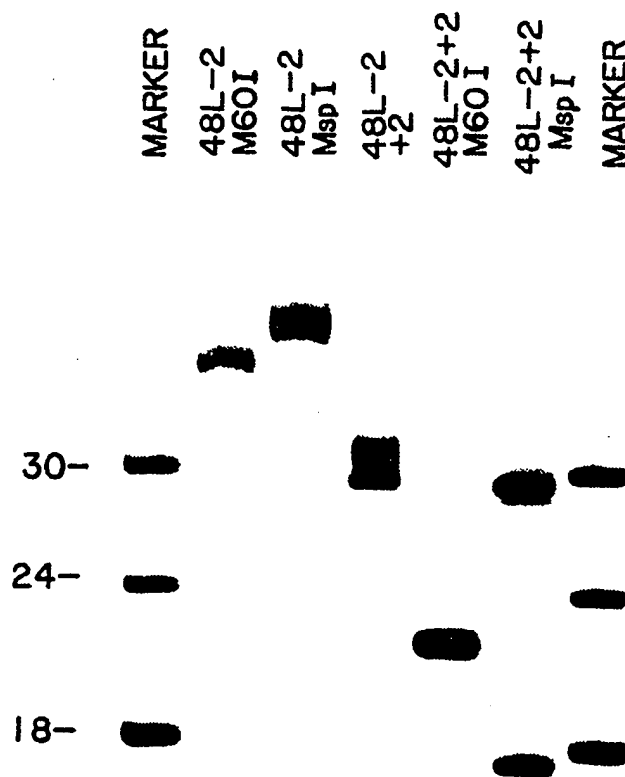


FIG. 3

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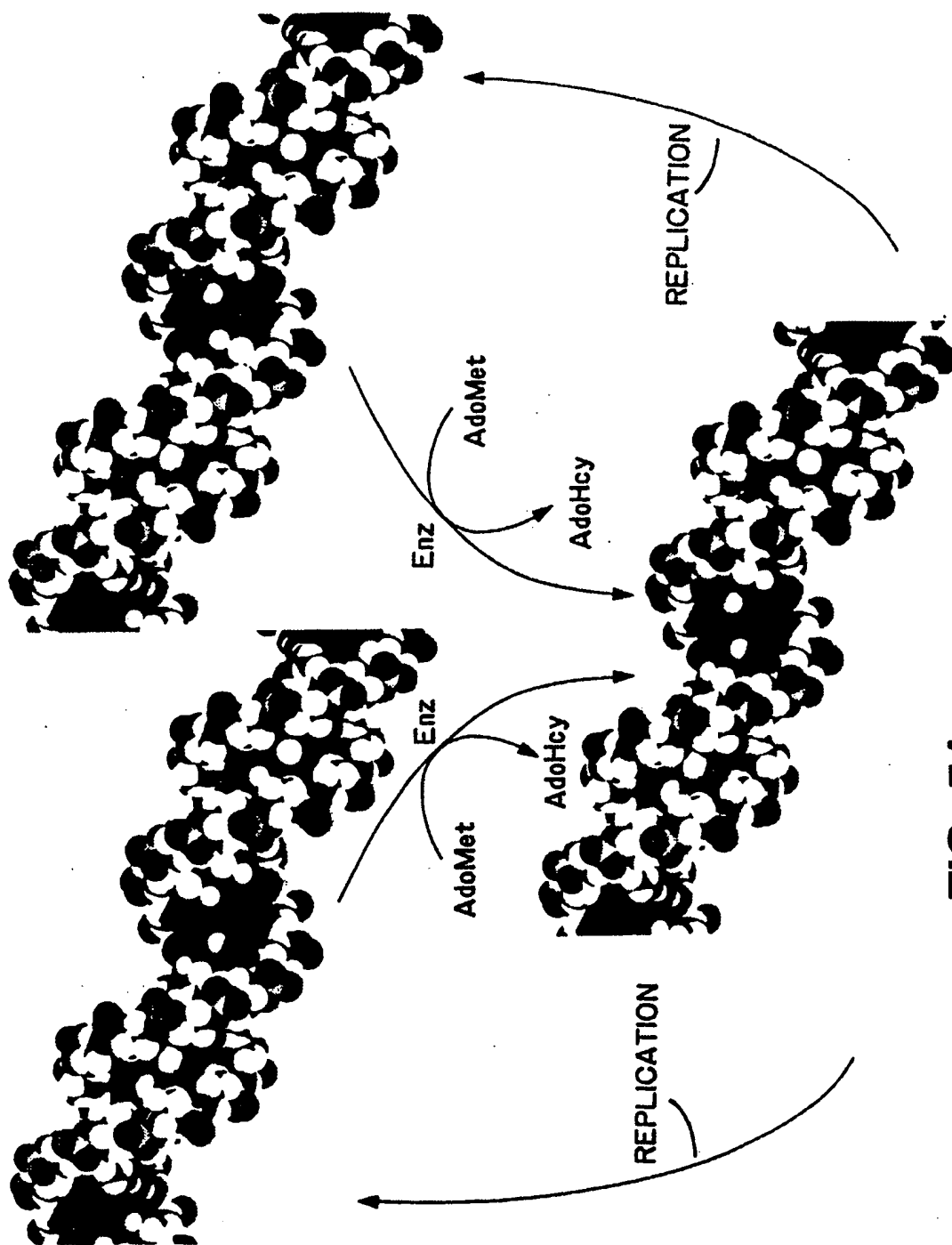
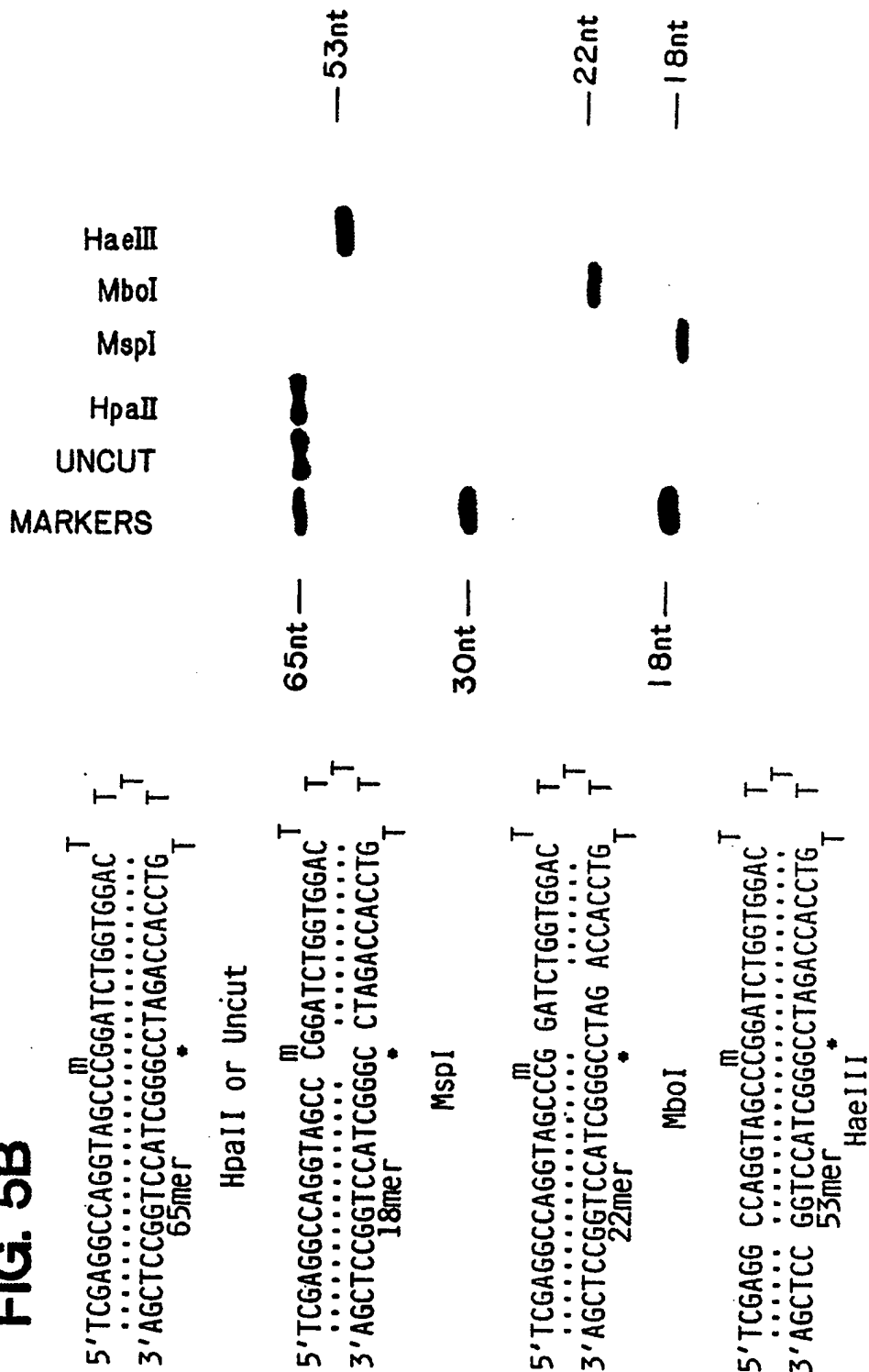
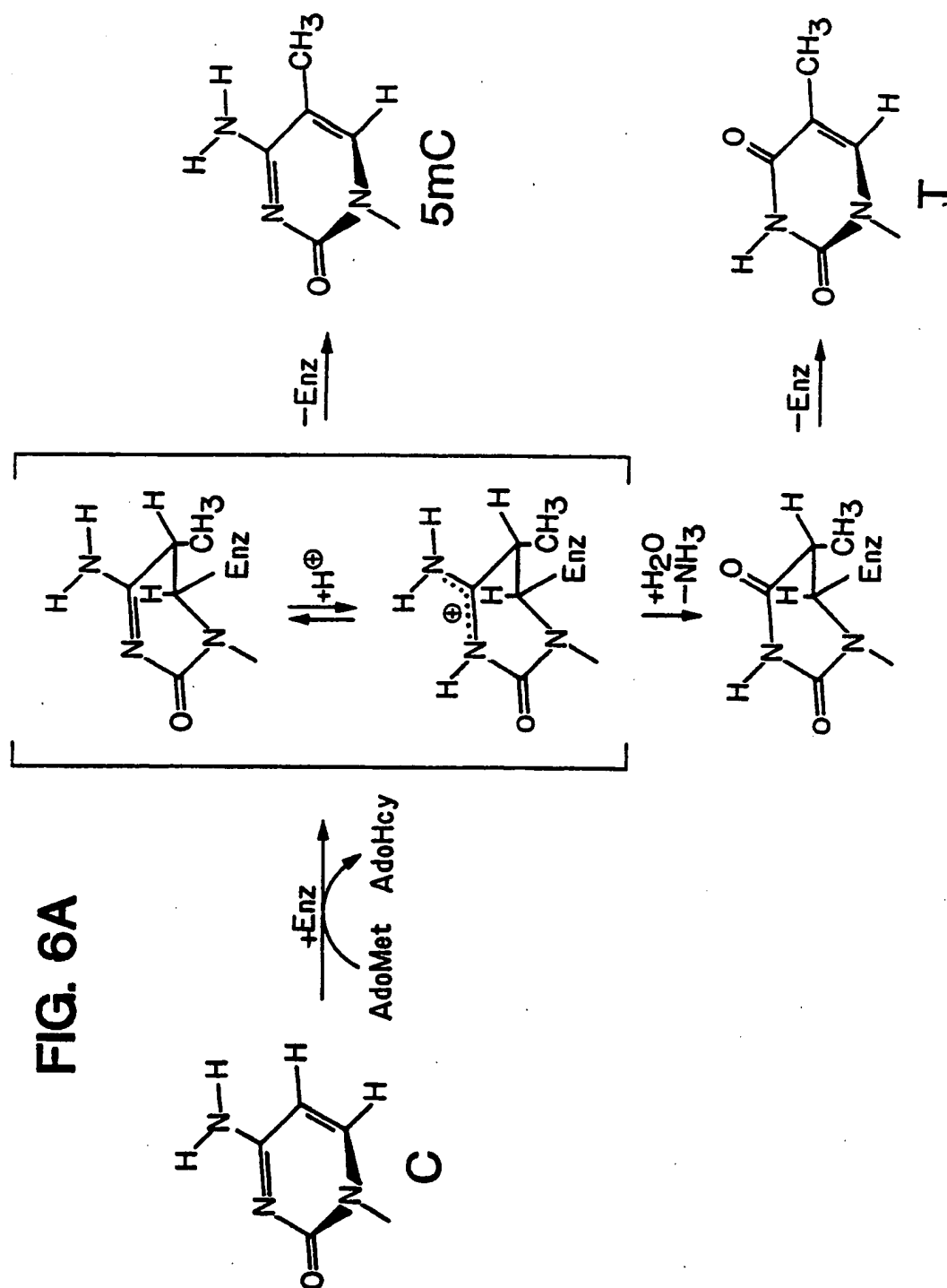


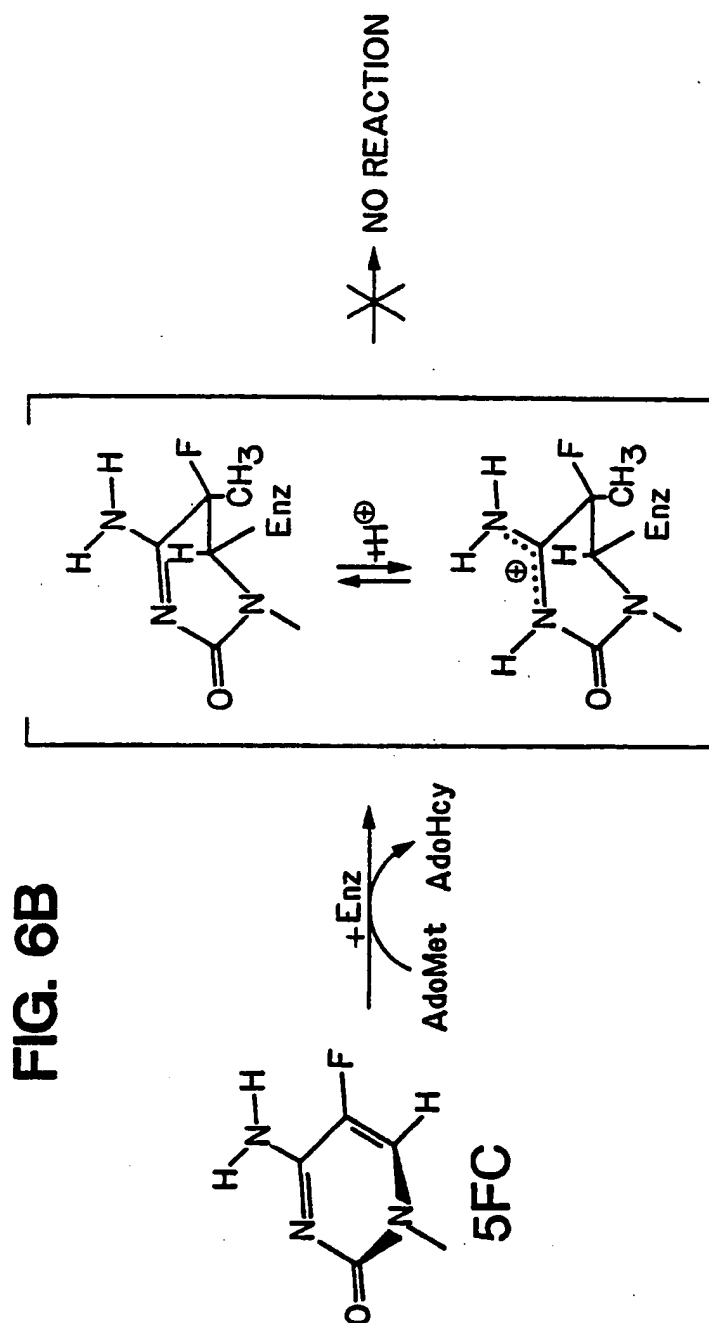
FIG. 5A



FIG. 5B

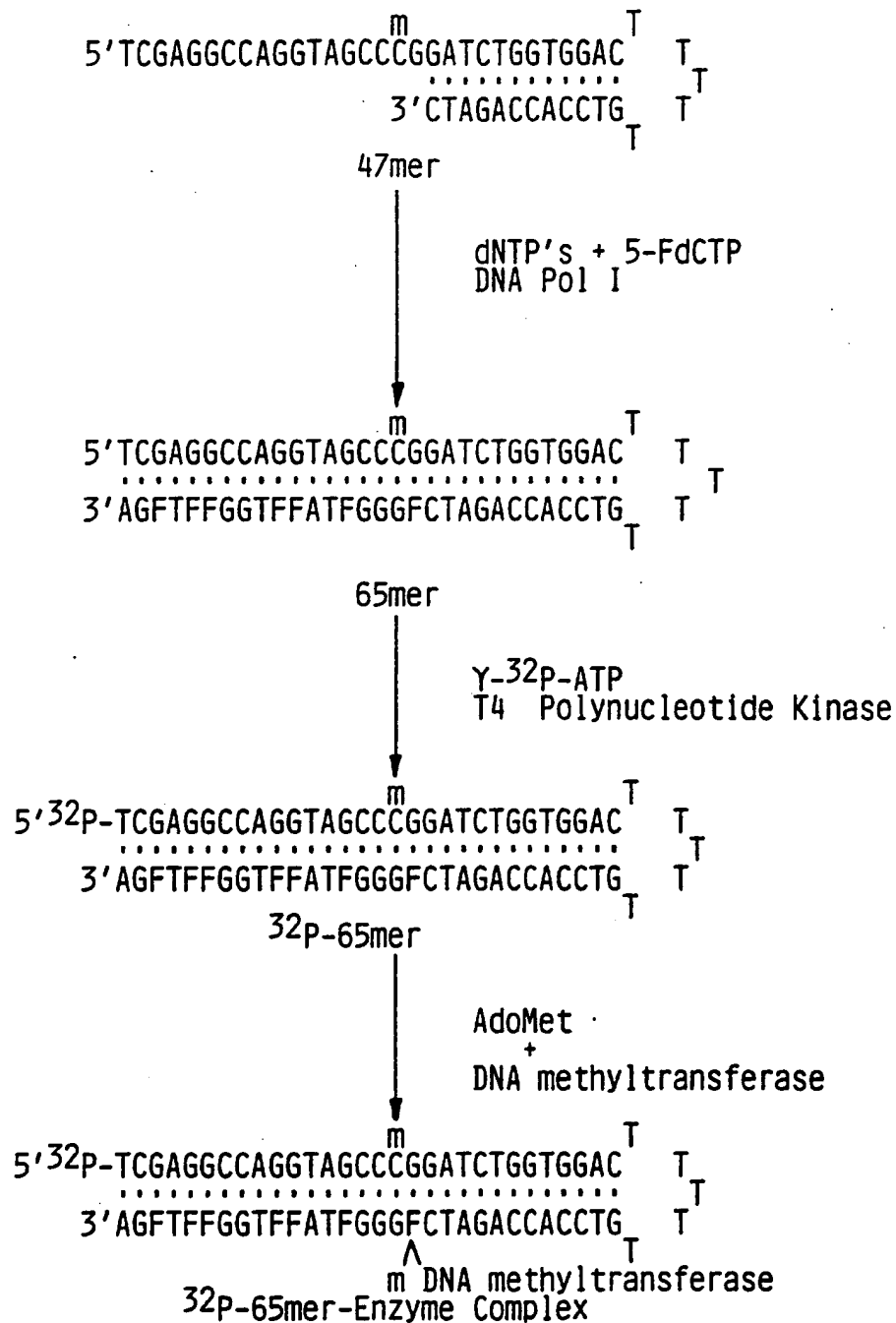






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FIG. 7A



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65mer+MTASE }  
+AdoMet }  
FdC65mer+MTASE }  
+AdoMet }

FIG. 7C

-228,200  
-116,000  
-109,600  
-70,300  
-44,100  
-27,900  
-18,100  
-15,500

"153kD" COMPLEX-  
"120kD" COMPLEX-

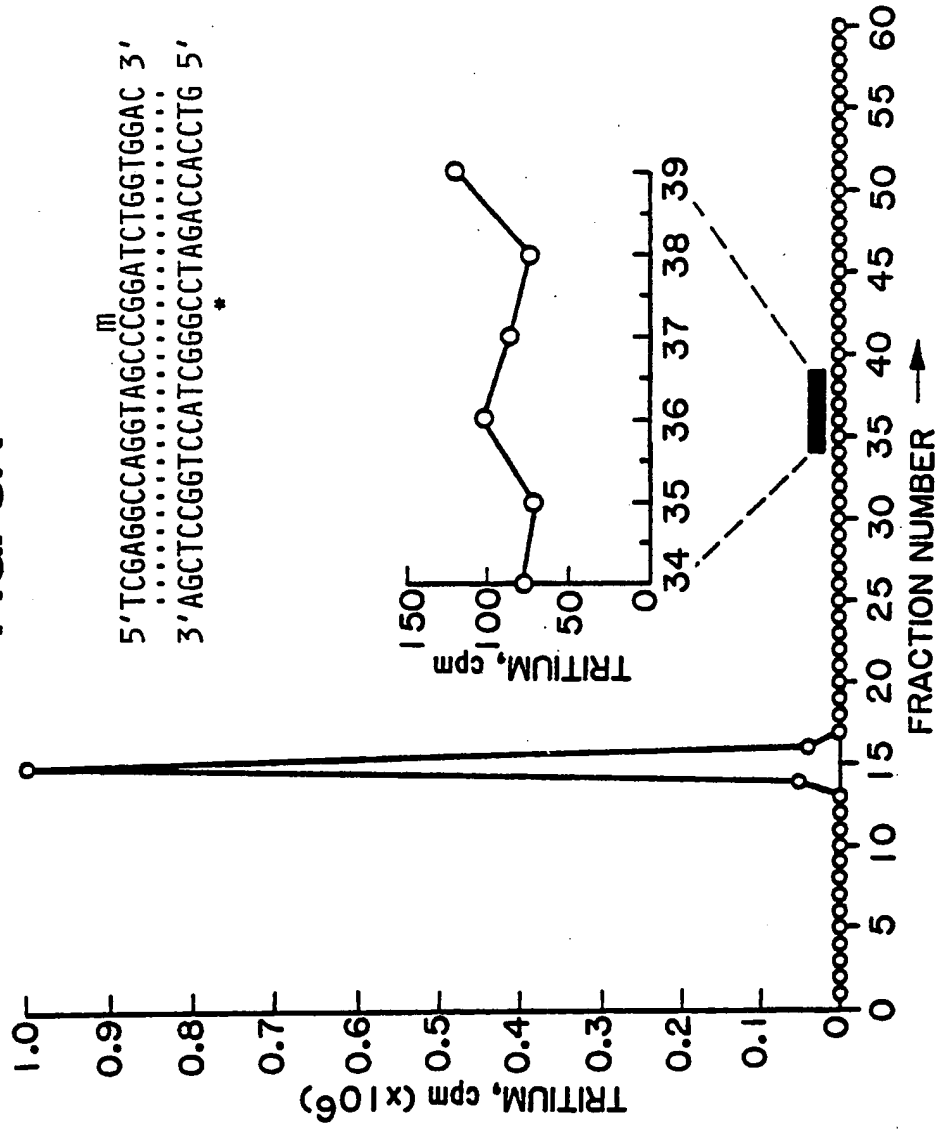
-228,200  
-116,000  
-109,600  
-70,300  
-44,100  
-27,900  
-18,100  
-15,500

MARKERS }  
65mer+MTASE }  
+AdoMet }  
FdC65mer+MTASE }  
-AdoMet }  
FdC65mer+MTASE }  
+AdoMet }

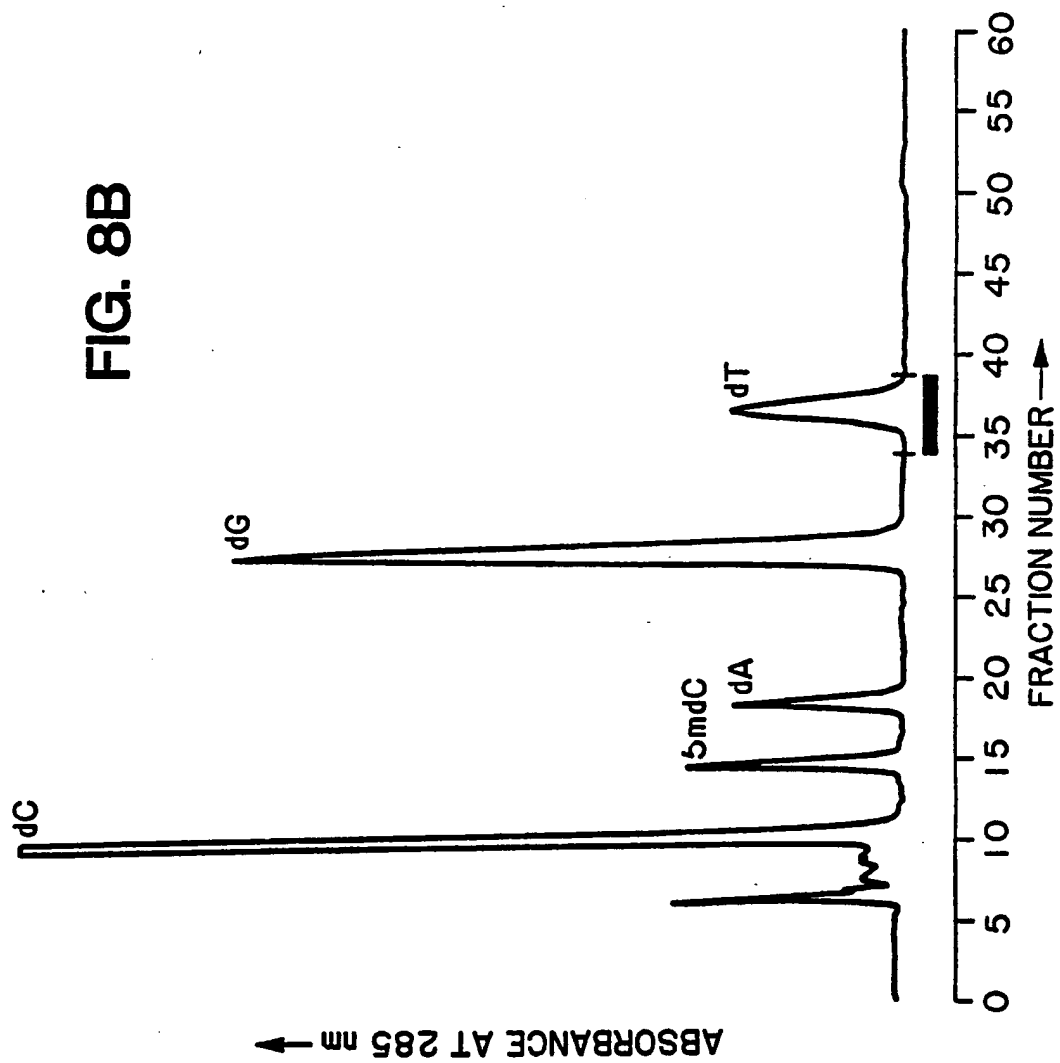
FIG. 7B

"153kD" COMPLEX-  
"120kD" COMPLEX-  
"79kD" COMPLEX-

FIG. 8A



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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07622

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C07H 1/00, 21/00 US CL : 536/27, 28, 29		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	536/27, 28, 29	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y, P	US, A, 4,990,499 (Gupta et al) 05 February 1991, see columns 7 and 8.	4, 5, 6
Y	US, A, 4,963,662 (Matthes et al) 16 October 1990, see column 3, lines 20-35.	1, 2, 3
Y, P	US, A, 5,002,868 (Jacobson et al) 26 March 1991, see entire document.	4, 5, 6
Y	US, A, 4,921,794 (Tabor et al) 01 May 1990, see entire document.	4, 5, 6
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
31 JANUARY 1992		12 FEB 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		James O. Wils n <i>James O. Wils n</i>